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REVIEW ARTICLE

New insights into the molecular mechanisms of the fibrinolytic system

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Summary. Fibrinolysis is regulated by specific molecular interactions between its main components. Activation of plasminogen by tissue-type plasminogen activator (t-PA) is enhanced in the presence of fibrin or at the endothelial cell surface. Urokinase-type plasminogen activator (u-PA) binds to a specific cellular u-PA receptor (u-PAR), resulting in enhanced activation of cell-bound plasminogen. Inhibition of fibrinolysis occurs at the level of plasminogen activation or at the level of plasmin. Assembly of fibrinolytic components at the surface of fibrin results in fibrin degradation. Assembly at the surface of cells provides a mechanism for generation of localized cell-associated proteolytic activity. This review includes novel proteins such a thrombin-activatable fibrinolysis inhibitor (TAFI) and discusses new insights into molecular mechanisms obtained from the rapidly growing knowledge of crystal structures of proteins.

Keywords: fibrinolysis, PAI-1, plasminogen, TAFI, t-PA, u-PA.

Introduction

The fibrinolytic system in mammalian blood plays an important role in the dissolution of blood clots and in the maintenance of a patent vascular system. The fibrinolytic system contains an inactive proenzyme, plasminogen, which can be converted to the active enzyme plasmin, which degrades fibrin into soluble fibrin degradation products (Fig.1). Two immunologically distinct physiologic plasminogen activators have been identified in blood: tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) [1]. Inhibition of the fibrinolytic system may occur at the level of plasminogen activation, mainly by a specific plasminogen activator inhibitor (PAI-1) or by thrombin-activatable fibrinolysis inhibitor (TAFI), and at the level of plasmin, mainly by α_2-antiplasmin. Some physicochemical properties of the main components of the fibrinolytic system are summarized in Table 1.

Tissue-type plasminogen activator-mediated plasminogen activation is primarily involved in the dissolution of fibrin in the circulation [2], whereas u-PA binds to a specific cellular u-PA receptor (u-PAR) resulting in enhanced activation of cell-bound plasminogen. The main role of u-PA is the induction of pericellular proteolysis during tissue remodeling and repair, macrophage function, ovulation, embryo implantation and tumor invasion [3].

Regulation and control of the fibrinolytic system is mediated by specific molecular interactions among its main components, and by controlled synthesis, release and clearance of plasminogen activators and inhibitors. Disorders of the fibrinolytic system may result from excessive activation (i.e. bleeding tendency) or from impaired activation (i.e. thrombotic complications) [4].

Protein structure of the main components

Most enzymes of the fibrinolytic system are serine proteases, because their active site consists of a 'catalytic triad' composed of the amino acids serine, aspartic acid and histidine. This active site is located in the C-terminal serine protease domain. The N-terminal region contains one or more functional domains, such as a finger domain (homologous to the fingers in fibronectin), an epidermal growth factor-like domain and a triple-loop structure called kringle domain. The main inhibitors of the fibrinolytic system are grouped into the serpin (serine protease inhibitor) superfamily. Serpins have in their C-terminal region a reactive center loop containing a specific peptide bond which is cleaved by their target enzyme, resulting

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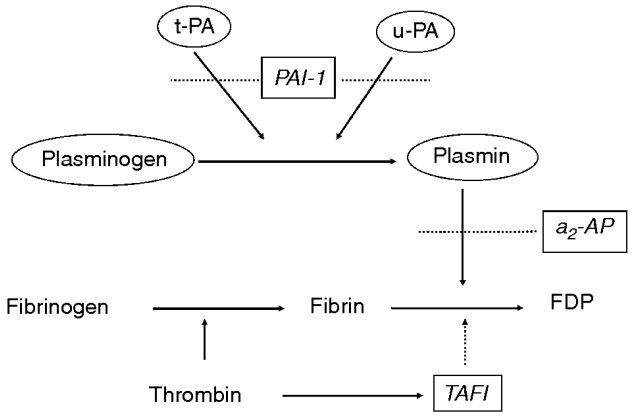

Fig. 1. Fibrin degradation by the fibrinolytic system. Plasminogen is activated by tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA). These enzymes are regulated by plasminogen activator inhibitor-1 (PAI-1). Plasmin degrades fibrin into soluble fibrin degradation products (FDP) and is regulated by α_2-antiplasmin (α_2-AP). Thrombin not only converts fibrinogen into fibrin, but also activates thrombin-activatable fibrinolysis inhibitor (TAFI), which inhibits fibrinolysis by modifying the fibrin substrate.

in the release of a peptide from the inhibitor and formation of an inactive enzyme-inhibitor complex. The active site of the enzyme in the complex is disrupted, which prevents the release of the cleaved inhibitor from the complex [5].

Plasminogen

Human plasminogen is a 92-kd, single-chain glycoprotein consisting of 791 amino acids; it contains 24 disulfide bridges and five homologous kringles. All plasminogen activators convert plasminogen to plasmin by cleavage of a single Arg561–Val562 peptide bond. The two-chain plasmin molecule is composed of a heavy chain containing the five kringles (N-terminal part of plasminogen) and a light chain (C-terminal part) containing the catalytic triad, composed of His603, Asp646 and Ser741 [6]. Native plasminogen has N-terminal glutamic acid ('Glu-plasminogen') but is easily converted by limited plasmic digestion to modified forms with N-terminal lysine, valine, or methionine, commonly designated 'Lys-plasminogen'. Glu-plasminogen exists in a closed (tight) conformation, whereas Lys-plasminogen presents a more open (relaxed) conformation, which is a preferential substrate for plasminogen activators [7]. The plasminogen kringles contain 'lysine binding sites' that mediate the specific binding of plasminogen to fibrin and the interaction of plasmin with α_2-antiplasmin; they play a crucial role in the regulation of fibrinolysis [8]. Low-molecular-mass variants of plasmin(ogen) include miniplasmin(ogen) and microplasmin(ogen), with only one kringle and no kringles at all, respectively, attached to the light chain.

Tissue-type plasminogen activator (t-PA)

t-PA is a 70-kd serine protease, originally isolated as a single polypeptide chain of 527 amino acids [9,10]. However, native t-PA contains an N-terminal extension of three amino acids (Gly-Ala-Arg-). Tissue-type plasminogen activator is converted by plasmin to a two-chain form by hydrolysis of the Arg275–Ile276 peptide bond. In contrast to the single-chain precursor form of most serine proteases, single-chain t-PA is enzymatically active [11]. The N-terminal region of t-PA is composed of several domains with homologies to other proteins: a finger domain, including residues 4–50; an epidermal growth factor domain consisting of residues 50–87; and two kringles, including residues 87–176 and 176–262. The region constituted by residues 276–527 represents the serine protease part with the catalytic site, composed of His322, Asp371 and Ser478. These distinct domains in t-PA are involved in several functions of the enzyme, including its binding to fibrin, fibrin-specific plasminogen activation, rapid clearance *in vivo* and binding to cellular receptors. Binding of t-PA to fibrin is mediated by the finger and the second kringle domains [12,13]. The t-PA molecule comprises three potential *N*-glycosylation sites at Asn117, Asn184 and Asn448 and an O-fucose attachment site at Thr61. Although full-length t-PA has not been crystallized so far, the global atomic structure on the basis of the nuclear magnetic resonance (NMR) and crystal structures of the separate domains is available [14].

Table 1 Some properties of the main components of the fibrinolytic system

Component	M_r	Number of amino acids	Catalytic triad of serine proteases	Reactive site of serpins	Plasma concentration ($\mu g\ mL^{-1}$)	(molarity)
Plasminogen	92 000	791	–	–	200	2 μM
Plasmin	85 000	±715	His603, Asp646, Ser741	–	–	
t-PA	70 000	530 (527)*	His322, Asp371, Ser478	–	0.005	70 pM
u-PA	54 000	411	His204, Asp255, Ser356	–	0.002	40 pM
u-PAR	55 000	313	–	–	–	
α_2-Antiplasmin	70 000	464	–	Arg376–Met377	70	1 μM
PAI-1	52 000	379	–	Arg346–Met347	0.02	400 pM
TAFI	60 000	401	–	–	5	75 nM

*The numbering of amino acid residues is usually based on the initially determined incorrect value. t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; u-PAR, urokinase-type plasminogen activator receptor; PAI-1, plasminogen activator inhibitor-1; TAFI, thrombin-activatable fibrinolysis inhibitor.

Urokinase-type plasminogen activator (u-PA)

Single chain u-PA (scu-PA) is a 54-kd glycoprotein containing 411 amino acids [15]. On proteolytic cleavage of the Lys158–Ile159 peptide bond by plasmin, the molecule is converted to a two-chain derivative (tcu-PA). The catalytic triad is located in the C-terminal polypeptide chain and is composed of Asp255, His204 and Ser356. The N-terminal chain contains an epidermal growth factor domain (residues 5–49) and one kringle domain (residues 50–131) which does not contain a lysine binding site, explaining why u-PA does not bind directly to fibrin. A low-molecular-mass tcu-PA (33-kd) is generated with a high concentration of plasmin by hydrolysis of the Lys135–Lys136 peptide bond after previous cleavage of the Lys158–Ile159 bond. Another low-molecular-mass form of u-PA (32-kd) is generated with specific metalloproteases by hydrolysis of the Glu143–Leu144 peptide bond. Thrombin inactivates scu-PA by cleaving the Arg156–Phe157 peptide bond, two residues prior to the activation site. This reaction is strongly accelerated by thrombomodulin [16]. As for t-PA, the available global atomic structure of full-length u-PA is based on the NMR and crystal structures of the separate domains [17].

Urokinase-type plasminogen activator receptor (u-PAR)

u-PAR, the specific cell surface receptor for u-PA, is a heterogeneously glycosylated protein of 50 to 60 kd, synthesized as a 313-amino-acid polypeptide, anchored to the plasma membrane by a glycosyl phosphatidylinositol (GPI) moiety. The u-PAR molecule is composed of three related structural domains, of which primarily the N-terminal domain is involved in binding u-PA via its growth factor domain [18]. However, crystal structures of soluble u-PAR in complex with an antagonist peptide [19] or with the N-terminal fragment of u-PA show that all three domains of u-PAR are involved in a composite binding site in the central cavity of the receptor to generate high-affinity binding of u-PA [20]. The latter study also shows substantial u-PA-induced conformational changes in u-PAR which explain interactions of u-PAR with cellular components. Ligands of u-PAR other than u-PA usually bind at the outer side of the receptor, as demonstrated for vitronectin [21].

α_2-Antiplasmin

α_2-Antiplasmin is a 70-kd, single-chain glycoprotein containing about 13% carbohydrate. The molecule consists of 464 amino acids including four Cys residues [22], but only two of them form a disulphide bridge [23]. α_2-Antiplasmin is a serpin with reactive site peptide bond Arg376–Met377. Its concentration in human plasma is about 70 $\mu g\ mL^{-1}$ (about 1 μM). α_2-Antiplasmin is synthesized primarily in a plasminogen-binding form that becomes partially converted in circulating blood to a less active form that possibly lacks the C-terminal end of the molecule. Multiple lysine residues in this part of the molecule contribute, perhaps in a zipper-like fashion, to its binding to the kringles of plasmin(ogen) [24,25]. An X-ray crystal structure of α_2-antiplasmin reveals that the C-terminal end of the molecule is located in close proximity to the serpin reactive site loop where it may act as a template to accelerate the plasmin–α_2-antiplasmin interaction [26]. The inhibitor is cross-linked via Gln14 to the fibrin α-chain when blood is clotted in the presence of calcium ions and factor (F) XIIIa. During circulation in plasma, α_2-antiplasmin undergoes proteolytic cleavage at Pro12–Asn13 by a novel protease called antiplasmin-cleaving enzyme (APCE), which is a soluble form of fibroblast activation protein, a proline-specific serine protease [27]. The cleaved form cross-links approximately 13 times more rapidly to fibrin during clot formation than the native form and protects the clot against fibrinolysis more efficiently. A single nucleotide polymorphism in the α_2-antiplasmin gene corresponding with either Arg or Trp as the sixth amino acid has been found. This polymorphism may be a functional one, because the Arg6-form of α_2-antiplasmin is cleaved by APCE 8-fold faster than the Trp6-form and is therefore incorporated into fibrin to a greater extent [28].

Plasminogen activator inhibitor-1 (PAI-1)

PAI-1 is a 52-kd, single-chain glycoprotein consisting of 379 amino acids and belongs to the serpin superfamily [29]. Active PAI-1 is spontaneously converted into an inactive 'latent' form (see below), but is stabilized by binding to S-protein or vitronectin via its somatomedin B domain [30]. The overall crystal structure (Fig. 2A) is very similar to the structures of other active inhibitory serpins and consists of three β-sheets (A, B, C), nine α-helices (A through I),and an exposed loop containing the reactive site peptide bond Arg346–Met 347 [31]. Structure studies on PAI-1 in solution show that the exposed loop is folded closer to the protein core than the crystal structures suggest [32]. The availability of structural information may facilitate the development of therapeutic inhibitors of PAI-1 [33].

Thrombin-activatable fibrinolysis inhibitor (TAFI)

TAFI or plasma procarboxypeptidase B is a single-chain 60-kd glycoprotein consisting of 401 amino acid residues [34]. The proenzyme is converted into activated TAFI (TAFIa) after cleavage of the Arg92–Ala93 peptide bond. Indirect evidence suggests that the 92-residue activation peptide remains non-covalently connected to the zinc-containing catalytic domain [35]. The activation peptide contains four *N*-glycosylation sites (Asn22, Asn51, Asn63 and Asn86) and the catalytic domain only one (Asn219), although the latter site may not be occupied [36]. A model of TAFI is based on the structure of human pancreatic procarboxypeptidase B, which is 42% identical to TAFI [37]. Crystal structures are recently available for human TAFI [38], bovine TAFI [39] and bovine TAFIa [40]. They provide a structural basis for the intrinsic enzymatic activity of

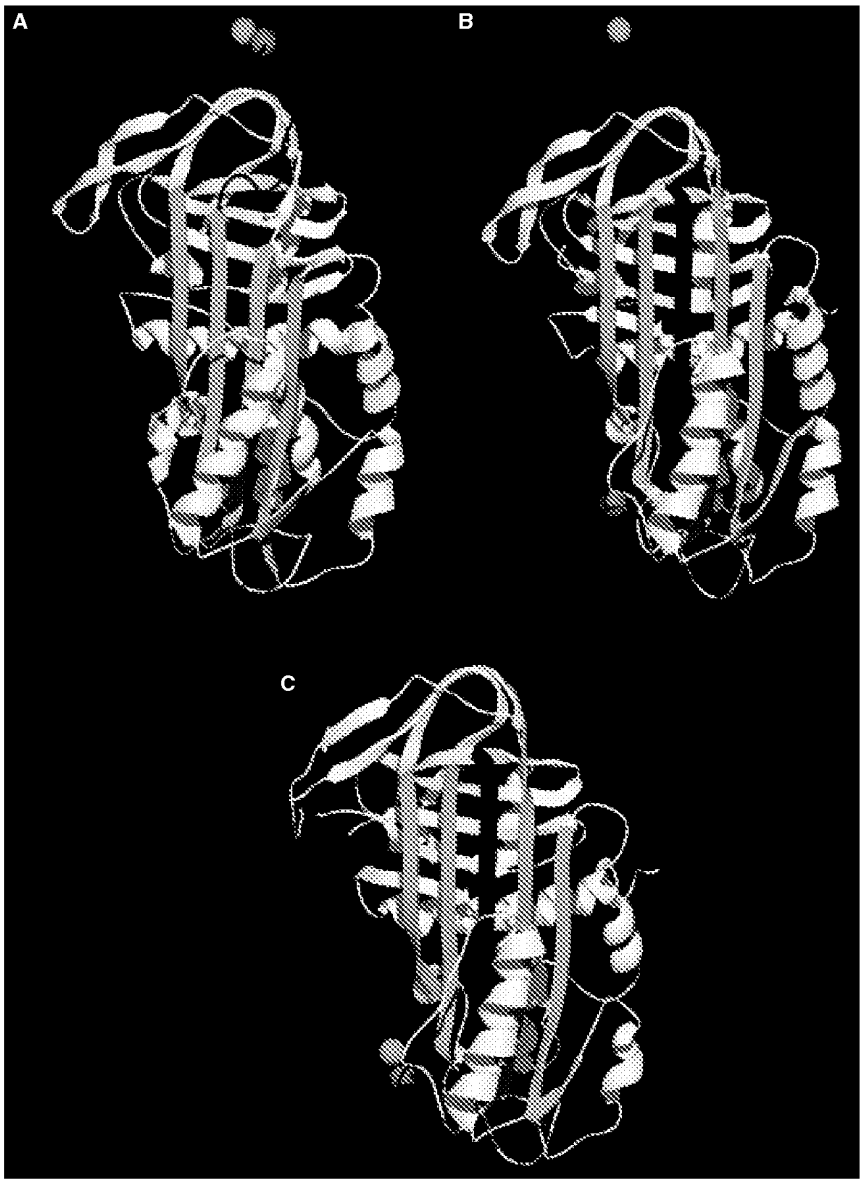

Fig. 2. The crystal structure of plasminogen activator inhibitor-1 (PAI-1) in the active (A), cleaved (B) and latent (C) conformation. β-Sheet A is indicated in green, the reactive site loop is indicated in red and the reactive site residues Arg346 and Met347 are represented as blue and yellow spheres, respectively. This illustration was originally published in Gils and Declerk *Thromb Haemost* 2004; **91**: 425–437 [33].

the proenzyme [39], although an antifibrinolytic role of this activity is questioned [41,42]. The crystal structures also point to one or two potential heparin-binding sites in TAFI and TAFIa [39,40].

Plasminogen activation by tissue-type plasminogen activator

In the presence of fibrin

In the absence of fibrin, t-PA displays a low activity towards plasminogen. In the presence of fibrin this activity is two orders of magnitude higher. Kinetic analysis suggests that the activation in the presence of fibrin occurs through the binding of an activator molecule to the fibrin clot surface and the subsequent binding of a plasminogen molecule to form a cyclic ternary complex [43]. Indeed, both t-PA and plasminogen have the capacity to bind to fibrin. This mechanism implies that fibrin fulfils a dual function, both as a cofactor of plasminogen activation and as a final substrate of generated plasmin.

Two phases can be distinguished in t-PA-induced lysis of a fibrin clot (reviewed in [44]). In the first slow phase, single-chain t-PA activates plasminogen on the intact fibrin surface. In the second phase, fibrin is partially degraded by plasmin and exposes additional and probably different (i.e. C-terminal as opposed to internal lysine residues) binding sites for plasminogen and possibly t-PA.

The molecular interactions that occur in the first phase are extensively reviewed by Medved and Nieuwenhuizen [45]. A set of specific low-affinity t-PA- and plasminogen-binding sites (Kd about 1 μM) is localized in each D region of fibrin(ogen). The tPA-binding site includes residues γ312–324 (present in FCB-5 fragment of fibrinogen) and the plasminogen-binding site includes residues Aα148–160 (present in FCB-2 fragment of fibrinogen). Crystal structures of the D region indicate that the binding sites of tPA and plasminogen are located close to each other (about 45 Å) and can facilitate the activation of plasminogen by t-PA. Additional studies also suggest the involvement of the fibrinogen αC-domains in the activation of plasminogen by t-PA. The recombinant Aα221-610 fragment, which corresponds to the αC-domain, binds both tPA and plasminogen with high affinity (Kd 16–33 nM) and efficiently stimulates the activation of plasminogen by t-PA. All t-PA and plasminogen binding sites in the D regions and αC-domains are cryptic in fibrinogen and become exposed in fibrin. The exposure of the binding sites in the αC-domains is connected most probably with their switch from an intramolecular interaction in fibrinogen to an intermolecular one in fibrin. Upon fibrin assembly, the interaction between the D and E regions probably causes conformational changes in the D regions that expose the binding sites [45]. The binding of the B knob in the E region to the βC-domain in the D region plays an important role in this process [46].

The generation of C-terminal lysine residues in partially-degraded fibrin in the second phase of clot lysis may result in a up to thirtyfold accumulation of plasminogen on the clot surface and a concomitant increase in lysis rate [47]. TAFI removes C-terminal lysine residues, resulting in a decreased accumulation of plasminogen and in an inhibition of the second phase of clot lysis [48].

Not only do molecular characteristics of fibrin(ogen), but also structural characteristics of the fibrin network regulate fibrinolysis [49]. Generally, clots that consist of thin fibers with many branch points are more slowly lyzed than clots that consist of thick fibers. However, there are several exceptions [46].

At the cell surface

A striking analogy exists between the role of fibrin and that of cell surfaces in plasminogen activation. Many cell types bind plasminogen activators and plasminogen, resulting in enhanced plasminogen activation and protection of bound plasmin from inhibition by α_2-antiplasmin.

Most cells bind plasminogen by its lysine binding sites with a high capacity but a relatively low affinity. Gangliosides, as well as a class of membrane proteins with C-terminal lysine residues such as α-enolase, play an important role in binding of plasminogen to cells [50]. Other plasminogen receptors include TIP49a [51], the integrin αMß2 [52] and histone H2B [53]. The latter plays a very prominent and functionally important role on macrophages [54]. The catalytic efficiency of t-PA for activation of cell bound plasminogen is about tenfold higher than in solution, possibly as a result of conversion of the plasminogen conformation to the more readily activatable 'Lys-plasminogen-like' structure [55] or of Glu-plasminogen to Lys-plasminogen [56]. Vascular cells have the capacity to regulate pericellular fibrinolysis by modulating the expression of plasminogen receptors; enhanced receptor occupancy results in enhanced plasminogen activation by t-PA [57].

A 36-kd membrane protein related to annexin A2 was proposed as the functional t-PA receptor on human umbilical vein endothelial cells (reviewed by Cesarman-Maus and Hajjar [58]). The receptor not only binds t-PA (Kd 30 nM), but also plasminogen (Kd 114 nM). Residues 8–13 (Leu-Cys-Lys-Leu-Ser-Leu) in annexin A2 are essential for binding of t-PA, whereas Lys302 appears to be crucial for binding of plasminogen. Annexin A2 accelerates plasminogen activation by t-PA in a purified system by sixtyfold and may play an important role in maintaining blood fluidity and non-thrombogenicity. In a rat carotid artery thrombus model, the patency of thrombosed carotid arteries is significantly enhanced by recombinant annexin A2 injection [59]. In addition, homozygous annexin α_2-null mice display deposition of fibrin in the microvasculature and incomplete clearance of injury-induced arterial thrombi [60] and auto-antibodies directed against annexin A2 are associated with thrombosis in patients with the anti-phospholipid syndrome [61]. Other studies show that annexin A2 heterotetramer consisting of two annexin A2 subunits and two S100A10 subunits accelerates plasminogen activation by t-PA stronger than annexin A2 alone [62]. They even suggest that not annexin A2 but the S100A10 protein represents the plasminogen and t-PA receptor, whereas annexin A2 serves to anchor S100A10 to the cell surface [63,64].

Cellular receptors may also play a role in the rapid clearance of t-PA from the circulation. Circulating t-PA (half-life of 5–6 min in humans) interacts with several receptor systems in the liver. Liver endothelial cells have a mannose receptor which recognizes the high mannose-type carbohydrate antenna on kringle 1, and liver parenchymal cells contain a calcium-dependent receptor that interacts with the finger and EGF domains of t-PA [65]. The latter receptor probably represents low-density lipoprotein receptor-related protein (LRP), which also recognizes t-PA/PAI-1 complexes [66,67].

In addition to its fibrinolytic role in the vasculature, t-PA may play different and important roles in the central nervous system (reviewed by Melchor and Strickland [68]). The plasminogen activator is expressed by neurons and microglial cells and may (i) generate plasmin to degrade a variety of non-fibrin substrates (e.g. β-amyloid [69]), (ii) act as a direct protease not involving plasminogen (e.g. for the activation of latent platelet-derived growth factor-CC [70]) or (iii) function as a non-proteolytic modulator (e.g. of the NMDA receptors [71]).

Plasminogen activation by urokinase-type plasminogen activator

In the presence of fibrin

In contrast to tcu-PA, scu-PA displays very low activity towards low-molecular-weight chromogenic substrates. Scu-PA appears to have some intrinsic plasminogen activating potential, which represents less than 0.5% of the catalytic efficiency of tcu-PA [72]. In plasma, in the absence of fibrin, scu-PA is stable and does not activate plasminogen; in the presence of a fibrin clot, scu-PA, but not tcu-PA, induces fibrin-specific clot lysis [73]. Scu-PA is an inefficient activator of plasminogen bound to internal lysine residues on intact fibrin, but has a higher activity towards plasminogen bound to newly generated C-terminal lysine residues on partially degraded fibrin. The fibrin-specificity of scu-PA does not require its conversion to tcu-PA, but is mediated by enhanced binding of plasminogen to partially digested fibrin [74]. Local conversion of scu-PA to tcu-PA by plasmin on the surface of fibrin during fibrinolysis, might however, contribute to the fibrin-specificity of scu-PA.

At the cell surface

The binding of scu-PA to u-PAR on the cell surface was claimed to be crucial for the activity of scu-PA under physiologic conditions [75]. Binding results in a strongly enhanced plasmin generation, as a result of effects on the activation of plasminogen and on the feedback activation of scu-PA to tcu-PA by generated plasmin. Both effects critically depend on the cellular binding of plasminogen. Cell-associated plasmin is protected from rapid inhibition by α_2-antiplasmin, which further favors the activation of receptor-bound scu-PA. This system can be efficiently inhibited by PAI-1 and PAI-2. A model based on u-PAR-dependent complex formation has been proposed, which would allow initiation of plasminogen activation by the low intrinsic activity of scu-PA [75]. Initiation of plasminogen activation may also involve other enzymes which can activate scu-PA to tcu-PA. These enzymes include matrix metalloproteases, such as MMP-2 [76] and several type II transmembrane serine proteases (TTSPs) such as matriptase [77], serase-1B [78] and hepsin [79].The observation that direct anchorage of u-PA to the cell surface (using a GPI-anchored u-PA mutant) leads to a potentiation of plasmin generation equivalent to that observed in the presence of u-PAR, suggests that u-PAR primarily functions to localize u-PA at the cell surface [80].

The u-PA/u-PAR system plays an important role not only in cancer [81], but also in vascular biology [82]. Plasminogen activation is often essential, although several plasminogen and protease-independent mechanisms, for instance, in cell signaling involve the u-PA/u-PAR system.

Although u-PAR is the main cellular receptor for u-PA, u-PA may be active on cells in a u-PAR-independent manner. One example involves the integrin αMß2 on neutrophils, which can bind both plasminogen and u-PA and thereby stimulate significantly the generation of plasmin [52].

Inhibition of plasminogen activators by PAI-1 and other serpins

PAI-1 is the primary inhibitor of both t-PA and u-PA. The serpin is present at a low concentration in normal plasma, but at higher concentrations in many clinical conditions [83]. Platelets contain relatively large amounts of latent PAI-1, but recent evidence suggests that platelets can synthesize active PAI-1 [84]. Reported second order rate constants for the inhibition of tPA by PAI-1 are in the order of 10^7 M^{-1} s^{-1}. Calculations show that single-chain tPA in plasma containing 0.02 μg mL^{-1} PAI-1 (0.4 nM) is 50% inhibited in 5 min. This is about equal to the half-life of tPA in the circulation with respect to liver uptake, indicating that both clearance systems act hand in hand to restrict the activity of tPA in the circulation.

PAI-1 reacts with single-chain and two-chain t-PA and with tcu-PA, but not with scu-PA [85]. PAI-1 inhibits its target proteinases by formation of a 1:1 stoichiometric reversible complex, followed by covalent binding between the hydroxyl group of the active site serine residue of the proteinase and the carboxyl group of the P1 residue at the reactive site ('bait region') of the serpin. Highly positively charged regions in t-PA (residues 296–304) [86] and in u-PA (residues 179–184) [87] are involved in the rapid interaction.

In addition to the active inhibitory conformation of PAI-1 (Fig. 2A), other conformations which are non-inhibitory have been described. The active form of PAI-1 spontaneously converts into the latent form (50% conversion in approximately 2 h at 37 °C), which is accompanied by large structural changes [88]. These changes involve the insertion of the N-terminal side of the exposed reactive site loop into β-sheet A, as well as a translocation of the P1-P1' peptide bond Arg346–Met347 to the opposite pole of the molecule and a distortion of the reactive site loop (Fig. 2C). The latent form can be reactivated *in vitro* and possibly *in vivo*. A 'substrate' form of PAI-1 does not form stable complexes with t-PA or u-PA, but is cleaved at the P1-P1' peptide bond [89]. Cleaved PAI-1 shows, just as latent PAI-1, the insertion of the N-terminal side of the reactive site loop into β-sheet A (Fig. 2B). The structural basis of the behavior of substrate PAI-1 is not yet known, but is somehow related to a specific transition state between the active and latent conformations [90]. Stabilization of this transition state by ligands bound to the α-helix F modulates the substrate behavior of PAI-1 [91].

The two-chain forms of t-PA and u-PA are also efficiently inhibited by plasminogen activator inhibitor-2 (PAI-2) [85], but the plasma levels of this serpin are normally low or even undetectable. Only a small percentage of PAI-2 is secreted and alternative roles for PAI-2 have been postulated [92]. T-PA and two-chain u-PA are slowly inhibited in plasma by α_2-macroglobulin, α_2-antiplasmin, α_1-antitrypsin, C1-inhibitor and

plasminogen activator inhibitor-3 (PAI-3, which is identical to activated protein C inhibitor).

The activity of t-PA in the central nervous system is not only regulated by PAI-1, but also by neuroserpin [93].

Inhibition of plasminogen activation by thrombin-activatable fibrinolysis inhibitor

TAFI is the most recently discovered fibrinolysis inhibitor that represents a link between coagulation and fibrinolysis [94,95]. The inhibitor is only slowly activated by thrombin, but the activation is accelerated one thousand two hundred and fiftyfold by thrombomodulin [96]. TAFI is both activated and inactivated by plasmin [97]. TAFIa is a plasma carboxypeptidase B [34] and is identical to carboxypeptidase U [98,99] and carboxypeptidase R [99,100]. The active enzyme is relatively unstable under physiological conditions (half-life about 10 min). Two naturally occurring variants of TAFI (Thr325 and Ile325) differ substantially after activation with respect to thermal stability and antifibrinolytic activity [101]. Crystal structures of TAFI (Fig. 3) suggest that the stability of TAFIa is regulated by the dynamics of a 55-residue segment (residues 296–350) that includes residues of the active site wall. Release of the activation peptide increases this dynamics, which results in conformational changes in TAFIa and disruption of the catalytic site [38]. Understanding the mechanism of inactivation of TAFIa is important for new therapeutic strategies for prevention and treatment of both bleeding and thrombosis.

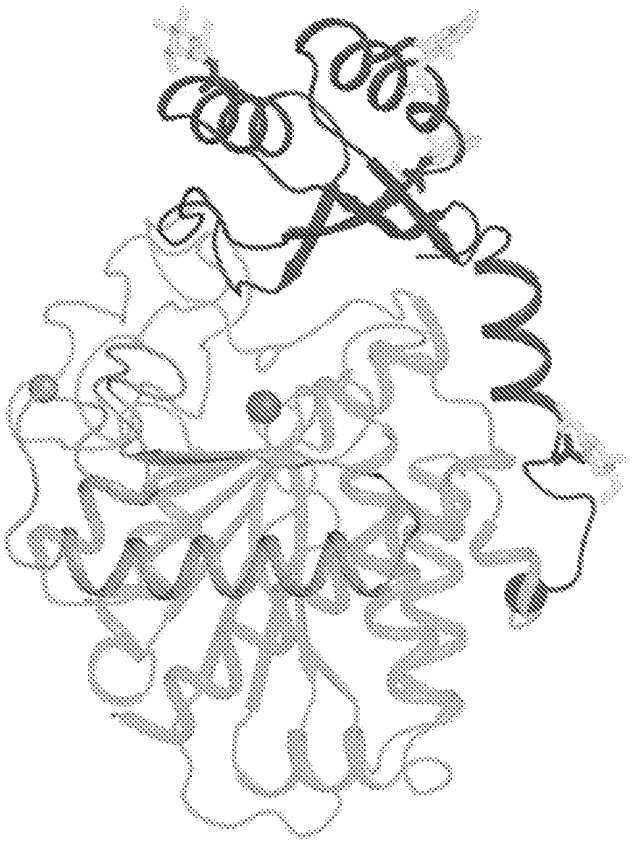

Fig. 3. The crystal structure of thrombin-activatable fibrinolysis inhibitor (TAFI) with the activation peptide in blue, the catalytic domain in green and the dynamic flap residues 296--350 in orange. The catalytic zinc ion is shown as a magenta sphere and the four N-linked glycans are shown in yellow stick representation. This research was originally published in *Blood*. P. F. Marx, T. Harma, C. Brondijk, T. Plug, R. A. Romijn, W. Hemrika, J. C. M. Meijers and E. G. Huizinga. Crystal structures of TAFI elucidate the inactivation mechanism of activated TAFI. *Blood* 2008; **112**: 2803–2809. © American Society of Hematology.

The antifibrinolytic activity of TAFIa is ascribed to the elimination of C-terminal lysine and arginine residues from partially degraded fibrin. This results in a strongly reduced binding of plasminogen and a concomitant reduction of the activation of plasminogen on the fibrin surface [48]. Other, probably secondary, consequences include a reduced conversion of Glu-plasminogen to Lys-plasminogen and a reduced protection of plasmin from inhibition by α_2-antiplasmin. TAFIa also cleaves C-terminal lysine and arginine residues from cellular receptors of plasminogen and inhibits cellular processes such as cell migration *in vivo* [102] and angiogenesis *in vitro* [103].

Inhibition of plasmin by α_2-antiplasmin

α_2-Antiplasmin forms an inactive 1:1 stoichiometric complex with plasmin. The inhibition involves two consecutive reactions: a fast, second-order reaction producing a reversible inactive complex, which is followed by a slower first-order transition resulting in an irreversible inactive complex. The second order rate constant of the inhibition is very high ($2–4 \times 10^7$ M^{-1} s^{-1}), but this high inhibition rate depends on the presence of free lysine binding sites and a free active site in the plasmin molecule and on the availability of a site complementary to the lysine binding site (plasminogen binding site) and of the reactive site peptide bond in the inhibitor. The half-life of plasmin molecules generated at the fibrin surface, which have their lysine binding sites and active site occupied, is two to three orders of magnitude longer than that of free plasmin [104]. The inhibition of miniplasmin and microplasmin by α_2-antiplasmin occurs slowly compared with intact plasmin.

α_2-Antiplasmin Enschede is an inactive mutant that does not form an irreversible complex with plasmin but is cleaved by plasmin such as a substrate [105]. The mutation involves an insertion of an alanine residue in the reactive center loop [106], which nicely illustrates that the serpin-inhibitory mechanism critically depends on the length of this loop [5].

Conclusion

The main components of the fibrinolytic system have been identified and mutual interactions between plasmin(ogen) and its activators, inhibitors, cofactors or receptors are becoming elucidated. Essential information originates from new protein structures, predominantly obtained by crystallography. These studies, along with studies on the regulation of the relevant genes, will further define the physiological and pathophysiological role of the fibrinolytic system.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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